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CARBON DIOXIDE EFFECTS ON LAG PERIODS IN BOD STUDIES

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September 1962

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FINAL REPORT

PROJECT NO. B-223

CARBON DIOXIDE EFFECTS ON LAG PERIODS IN BOD STUDIES

By

PETER E. GAFFNEY

SEPTEMBER, 1962

GRANT-IN-AID WP-211 (C1)
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Final Report, Project No. B-223

SUMMARY

The investigations described in this report were conducted under National Institutes of Health Grants RG-7549 and WP-211 (C1) covering the period October 1, 1960 to September 30, 1962. The work represents an effort of one-man year on the part of the project director in addition to a limited amount of student assistance.

The objective of the research was to determine the effectiveness of carbon dioxide in reducing or eliminating lag periods during the oxidation of pure organic substrates and sewage by mixed microbial cultures.

The results indicate that no benefit would be derived from supplementing Standard Dilution Biochemical Oxygen Demand (BOD) water with carbon dioxide. It is concluded that a better approach to reliable short-term BOD tests would be the use of mass washed cell inocula.

However, the results do indicate that the absence of carbon dioxide in Warburg BOD tests may affect the validity of results obtained by this method.

I. INTRODUCTION

The objective of the research reported herein was to determine quantitatively the effect of the presence or absence of carbon dioxide on lag periods observed in biochemical oxygen demand (BOD) tests. That such lag periods exist has previously been shown in studies on the lower fatty acids (1) where it was found difficult to eliminate the lag periods even when adapted seed was used. Walker (2) was the first to show a definite effect of carbon dioxide on early lag periods in the growth of bacteria in pure culture, and it was therefore necessary to extend his study into an investigation of the effect on mixed cultures of inocula as employed in the BOD tests.

II. EXPERIMENTAL

The investigation was divided into three phases in order to accomplish the following related objectives: (1) determination of carbon dioxide effects on relatively short lag periods with low substrate concentration in the Standard Dilution BOD method, (2) determination of carbon dioxide effects on high substrate concentrations (1000 mg/l) measured by chemical oxygen demand (COD) removal, and (3) determination of carbon dioxide effects on high substrate concentrations using the direct Warburg BOD method.

The procedures employed in these tests were performed according to those outlined in Standard Methods For the Examination of Water and Wastewater (3). Techniques applicable to the specific experiments are described in the appropriate subsections on the following pages.

A. Carbon Dioxide Effects on Glucose COD Dissimilation

The results obtained by Walker (2) in 1932 indicated that the phenomenon of early lag in pure cultures of Escherichia coli was due largely, if not

entirely, to the time required by the cultures to build up the carbon dioxide content of the medium to a level essential for growth. This focused attention on the possibility of a definite carbon dioxide requirement for heterotrophs. In an attempt to explain this requirement, Werkman and Wood (4) postulated a condensation of pyruvate and carbon dioxide to form oxalacetic acid and indicated its critical importance with respect to the citric acid cycle. Krampitz and Werkman (5) later showed some support for this and more definite evidence on an enzymatic basis was later shown by Kaltenbach and Kalnitsky (6). In 1958, Field and Lichstein (7) reported that autoclaved glucose medium produced an unidentified factor which satisfied the carbon dioxide requirements of propionic-bacteria for early initiation of growth.

The objective of this phase of the investigation was to determine the effect of carbon dioxide tension on the initiation and rate of glucose catabolism by mixed cultures (sewage inocula) in a dispersed aeration system.

The medium consisted of basal salts plus phosphate buffer (pH 7.0) plus 1000 mg/l glucose and was inoculated (1.0 per cent by volume) with various settled domestic sewage samples. The mixtures were divided into three equal aliquots to receive different air supplies. Fritted glass bubblers were inserted into the system between the main air supply and the growth flasks (Figure 1). The bubblers contained either water, a supersaturated solution of sodium bicarbonate, or 20 per cent potassium hydroxide so that the air delivered to the growth flasks was normal, carbon dioxide-supplemented, or carbon dioxide-free, respectively. Carbon dioxide-free air was demonstrated by precipitation tests with barium hydroxide. In three of the tests a carbon dioxide gas tank was used for supplementation instead of the bicarbonate solution.

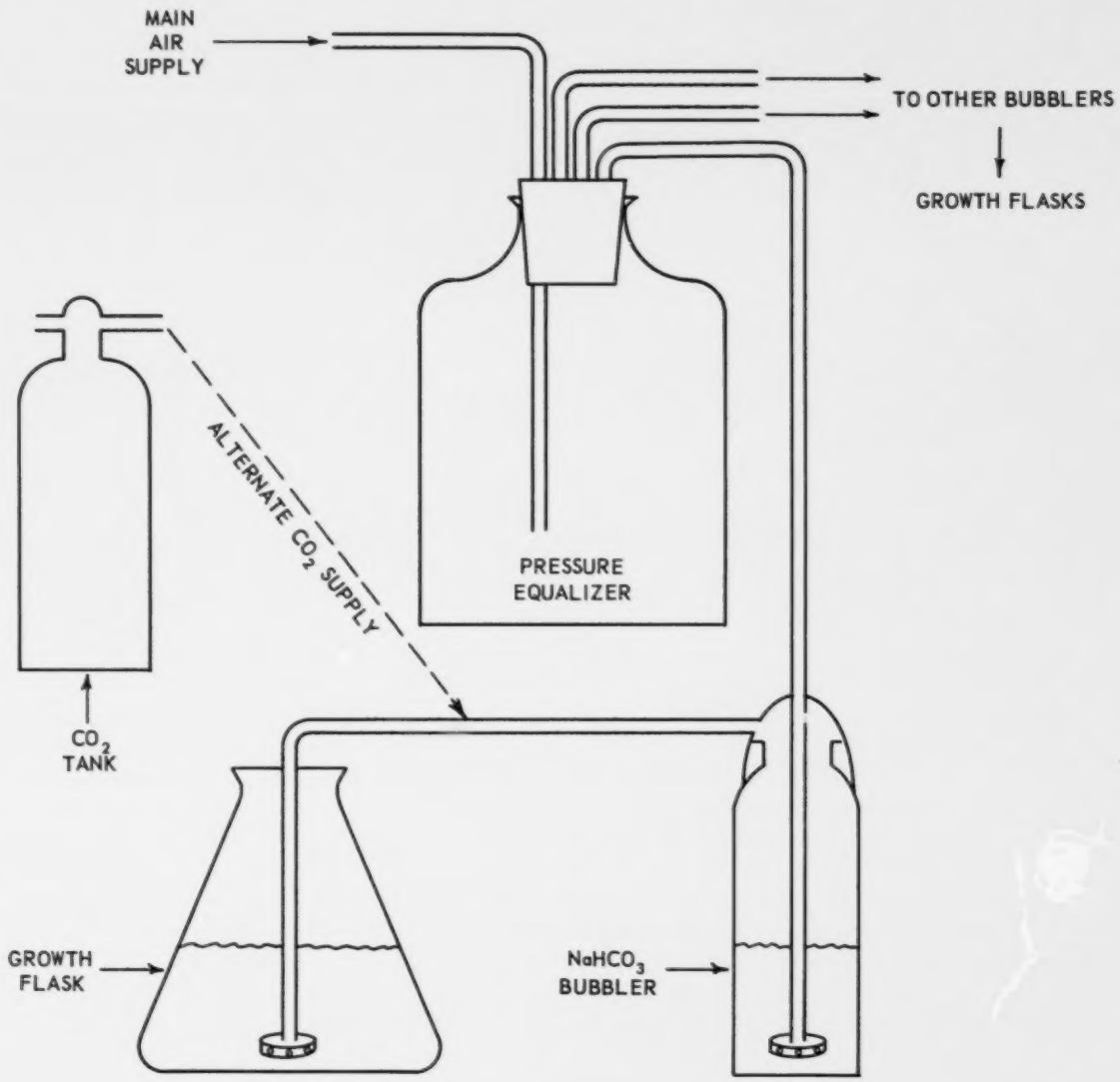

FIGURE 1. GAS SUPPLY SYSTEM

Glucose catabolism was measured daily by analysis of COD according to the 11th edition of Standard Methods.

The tests were run with five different sewage inocula and the results are shown in Table I. The COD values are averages of triplicate analyses and the experimental error was ±4.0 per cent. During test Number IV, after aeration for one day, total bacterial plate counts (on glucose agar) were made on the solutions from the three growth flasks. The buffer held the pH in each test between 6.9 and 7.2. Only the results after one day's aeration are given because the early period is of most interest.

The data from the first four tests consistently show that a greater percentage of glucose COD was destroyed after one day when carbon dioxide was added or left in the air supply and less was destroyed when carbon dioxide-free air was supplied. Also, the growth activity (in terms of numbers of organisms) followed the same pattern. The COD results of test Number V does not follow that pattern and in this test, carbon dioxide had no effect on the one-day values. The data in Table II are derived from those in Table I. Here it is shown that, compared with carbon dioxide-free air, normal air allowed for an additional 55 mg/l of glucose COD dissimilated in the first day and carbon dioxide-enriched air resulted in an average increase of 284 mg/l.

The complete 7-day curves during test Number II are presented in Figure 2. It can be seen that the bulk of catabolic activity occurred during the first day with carbon dioxide-supplemented air, during the second day with normal air, and not until the third day with carbon dioxide-free air.

It can be concluded that increased carbon dioxide tension results in a greater initial rate of glucose catabolism and growth. Different sewage samples show a variable response to the presence of carbon dioxide.

TABLE I

AMOUNT OF GLUCOSE C.O.D.[+] DISSIMILATED
AND BACTERIAL COUNT AFTER AERATION FOR ONE DAY

TEST NO.	WATER		BUBBLER $NaHCO_3$		KOH	
	mg./L.	%	mg./L.	%	mg./L.	%
I	390	34.0	490	43	210	18
II	50	4.4	670	58	0	0
	WATER		CO_2 GAS		KOH	
	mg./L.	%	mg./L.	%	mg./L.	%
III	40	3.5	140	12	30	2.6
IV	60	5.2	380	33	10	0.9
V	12	1.0	13	2	25	3.0
	NO. ORGANISMS PER ML.					
IV	34,000,000		80,000,000		15,000,000	

[+] Initial Glucose = 1000 mg./L.; initial C.O.D. = 1150 mg./L.

TABLE II

INCREASED GLUCOSE C.O.D. DISSIMILATED WITH NORMAL AND CARBON DIOXIDE-ENRICHED AIR AS COMPARED WITH CARBON DIOXIDE-FREE AIR

Test No.	Normal Air	CO_2-Enriched Air
	mg/L	mg/L
I	+ 180	+ 280
II	+ 50	+ 670
III	+ 10	+ 110
IV	+ 50	+ 370
V	- 13	- 12
Mean Increase	+ 55	+ 284

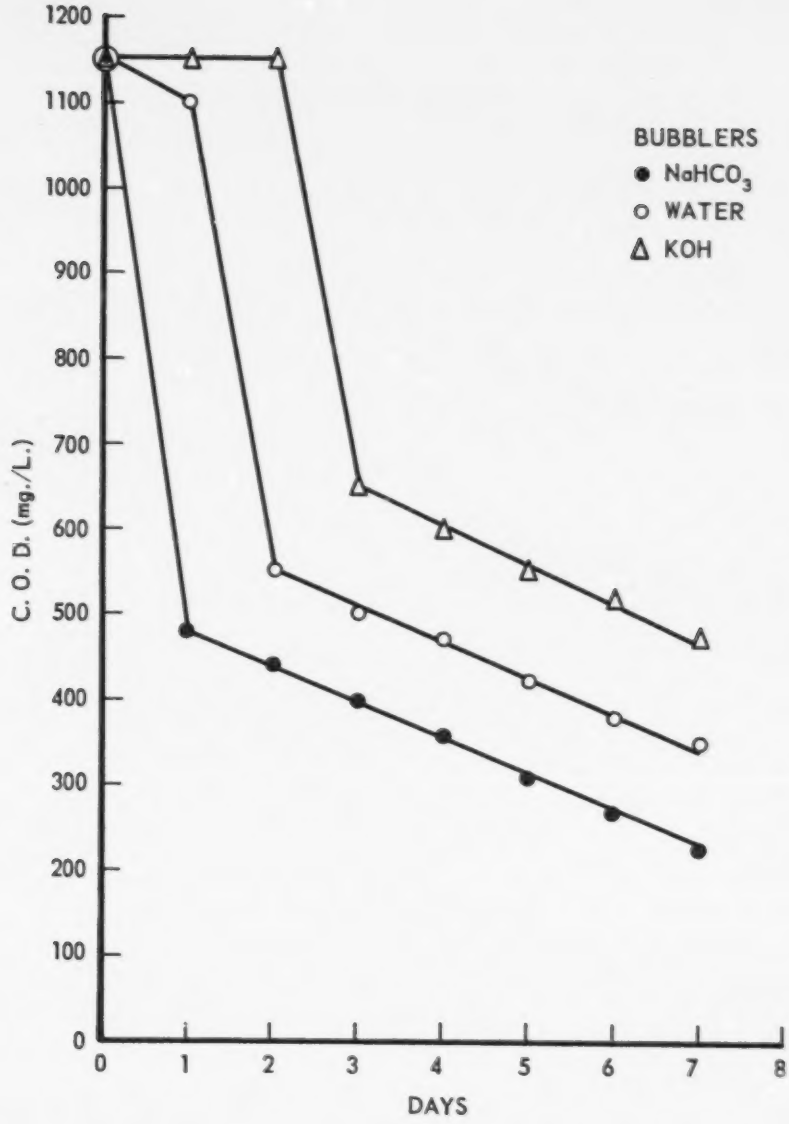

FIGURE 2. DISSIMILATION OF 1000 mg./L. GLUCOSE

B. Standard Dilution BOD Tests on Various Substrates

The objective of this phase of this project was to determine the effect of bicarbonate addition (to the dilution water) on the lag or initial oxygen-demand period observed with various substrates (sewage, glucose, and glucose-glutamic acid mixture).

Standard dilution water was used except where specified by modification with bicarbonate or substitution of ammonia-nitrogen with nitrate-nitrogen. Where pure substrates were used, the water was seeded with 0.1 per cent (by volume) settled domestic sewage. The seed in these tests, as determined by separate analyses, had a 5-day BOD of between 0.1 and 0.3 ppm.

In Tables III and IV are shown the results of the tests with sewage as the substrate. One hundred parts per million bicarbonate did not increase oxygen demand in the initial period. With ammonia as the nitrogen source, the presence of bicarbonate apparently increased the amount of nitrification in the later period (5 to 10 days). The particular sewage sample used in the test with nitrate exerted a high oxygen-demand in the early period. Nitrification was not observed in this test.

TABLE III

Effect of 100 PPM Bicarbonate on Sewage BOD Using Standard Dilution Water

Hours	Without Bicarbonate (PPM)	With Bicarbonate (PPM)
5	6	5
14	15	14
24	49	66
48	76	95
75	104	125
118	114	137
240	150	190

TABLE IV

Effect of 100 PPM Bicarbonate on Sewage BOD Using Modified (Nitrate) Dilution Water

Hours	Without Bicarbonate (PPM)	With Bicarbonate (PPM)
15	80	80
22	85	90
39	115	110
46	120	120
70	150	160
94	165	175
118	185	180
159	195	200
240	210	230

The results of experiments to determine the effect of bicarbonate on the BOD of glucose are presented in Tables V to X.

In Table V it is shown that bicarbonate concentrations of 10 to 100 ppm had no effect on the BOD exerted.

In Table VI the BOD at 1 and 2 days is shown to be higher in the presence of 1500 to 3000 ppm bicarbonate. However, it was observed that when acid was added to these bottles for the Winkler dissolved oxygen determination, there was a great amount of effervescence representing carbon dioxide evolution due to the acidic conditions. In this experiment the BOD of the test solutions was determined by subtracting the dissolved oxygen (DO) value of the test solution from that of a water blank with no bicarbonate. Thus the amount of iodine (equivalent to the oxygen content) scrubbed out of solution with the carbon dioxide upon the addition of the acid was an unknown value in this test.

In the next experiment, the results of which are presented in Table VII, the amount of oxygen equivalent released chemically was determined. BOD values were calculated by subtracting the DO of the test solution from that of an

TABLE V

Effect of 10 to 100 PPM Bicarbonate on BOD of 10 PPM Glucose

Days	ppm Bicarbonate				
	0 ppm	10 ppm	20 ppm	50 ppm	100 ppm
1	0	0	0.3	0	0
2	3.7	3.4	3.2	2.9	2.7
3	4.0	4.0	4.0	4.0	4.2
4	4.0	4.0	4.1	4.1	4.2
5	4.2	4.0	4.3	4.3	4.3

TABLE VI

Effect of 1000 to 3000 PPM Bicarbonate on BOD 10 PPM Glucose

Days	ppm Bicarbonate				
	0 ppm	1000 ppm	1500 ppm	2000 ppm	3000 ppm
1	0	0	0	0.2	2.4
2	0	0	1.6	2.0	4.6
3	3.3	3.8	4.6	5.1	5.3
4	3.5	4.5	5.0	5.1	5.3
5	4.0	5.4	5.0	5.1	5.3

aliquote of the same solution at the beginning of the test. Analyses were also made for pH and numbers of organisms. Enumeration of bacteria was obtained by pipetting ten-fold diluted portions of the test solution onto the surface of 1000 ppm glucose-agar plates. The plates were incubated at 37°C for one hour with the lids tipped in order to evaporate the moisture. With the lids on, the plates were then inverted and incubated at 20°C for 48 hours after which colonies were counted. Values presented are the average of at least two diluted portions.

The results in Table VII indicate that the presence of 1000 ppm bicarbonate enhanced cell division between 18 and 24 hours in conjunction with a slightly higher oxygen demand. The cells also reached greater total numbers in the presence of 1000 ppm bicarbonate but the greatest incremental increase in oxygen-demand occurred in each case between 24 and 48 hours.

With respect to the increase in numbers of organisms, the presence of 5000 ppm bicarbonate had a negative effect although there was a slight increase in the BOD rate compared to the test with no bicarbonate. During this experiment it was also observed that acidification with carbon dioxide release resulted in a corresponding release of oxygen equivalent to the extent of 0.5 to 0.6 ppm in the 1000 ppm bicarbonate tests and 3.6 to 4.8 ppm in the 5000 ppm tests.

TABLE VII

Effect of 1000 and 5000 PPM Bicarbonate on the BOD of 8.0 PPM Glucose Corrected For Oxygen Loss Due to Chemical Manipulation in the Tests

	ppm Bicarbonate								
	0			1000			5000		
Hours	No./ml	OD ppm	pH	No./ml	OD ppm	pH	No./ml	OD ppm	pH
6	20T	0.1	6.8	25T	0.2	7.2	28T	0	7.4
12	260T	0.3		220T	0.5		200T	0.6	
18	350T	0.8	6.8	600T	1.3	7.5	430T	1.1	7.8
24	12M	0.9		58M	1.5		15M	1.4	
48	4M	3.3	6.7	34M	3.6	7.6	8M	2.3	8.0
120		4.0			4.5			2.3	
240		4.3			5.6			3.5	

T = thousand; M = million

It is possible that pH differences due to the presence of bicarbonate may have been responsible for the differences in oxygen demand and cell growth. Therefore, the experiment was repeated using 2 ml of buffer per liter of dilution water (instead of 1.0 ml) and also adjusting (with sodium hydroxide) the control series (bottles with no bicarbonate) to a pH of 7.9 at the beginning of the test in order to have more comparable conditions. The results are presented in Table VIII.

The data in Table VIII show that with the procedure employed it was not possible to maintain comparable pH values throughout the period of the test. Although the presence of 3000 ppm bicarbonate, possibly due to the pH effect, enhanced the growth rate of the organisms there was no significant effect on the oxygen-demand rate. With 5000 ppm bicarbonate the oxygen-demand rate was somewhat inhibited.

TABLE VIII

Effect of 1000 to 5000 PPM Bicarbonate on the BOD of 10 PPM Glucose at Higher pH

	ppm Bicarbonate							
	0		1000		3000		5000	
Hours	OD ppm	pH	OD ppm	pH	OD ppm	pH	OD ppm	pH
6	0	7.9	0	7.7	0	8.0	0	8.0
12	0		0		0		0	
18	0.2		0.2		0.2		0	
24	0.4	7.8	0.3	8.2	0.2	8.4	0.4	8.4
48	4.3		4.5		3.1		2.8	
72	4.5		4.6		3.3		3.0	
120	6.5		6.5		3.3		3.9	
168	6.8	6.8	7.0	8.0	4.9	8.2	4.2	8.2
216	7.0		7.0		5.0		4.2	

	Number of Organisms per Ml	
Hours	No bicarbonate	3000 ppm bicarbonate
0	22,000	22,000
6	280,000	630,000
48	8,000,000	13,000,000

By increasing the volume of buffer in the BOD dilution water ten-fold (to 10 ml per liter) it was possible in subsequent tests to maintain pH between 7.0 and 7.3. The data shown in Table IX were obtained using the jug dilution method to determine the effect of 100 ppm bicarbonate on the BOD of 10 ppm glucose in standard and modified (nitrate) dilution water. The presence of bicarbonate did not reduce the lag period. Rather, it seemed to have a slight inhibiting effect on the oxygen-demand rate, especially as indicated by the 15 hour values. Although the 120 hour values are comparable, it is interesting to note the unexplained delay in oxygen uptake between 41 and 94 hours in the standard dilution water control test (no bicarbonate) as compared with the steady BOD increase observed in the other tests.

TABLE IX

Effect of 100 ppm Bicarbonate on BOD of Glucose in Standard and Modified Nitrate Dilution Water

Hours	Standard Dilution Water		Nitrate Dilution Water	
	No bicarbonate	100 ppm bicarbonate	No bicarbonate	100 ppm bicarbonate
	ppm	ppm	ppm	ppm
4.0	0.2	0.4	0.7	0.5
6.5	1.0	0.5	1.0	0.5
9.5	1.0	0.8	1.1	0.6
15.0	2.8	1.7	3.2	1.6
18.5	3.1	2.8	3.2	3.3
24.0	3.5	3.8	3.8	3.8
31.0	3.6	4.3	4.0	4.4
41	3.9	4.5	4.2	4.6
46	4.1	5.2	5.7	5.3
70	4.8	6.3	6.4	6.2
94	4.8	6.3	6.4	6.2
112	6.1	6.8	6.8	6.6
120	6.1	6.8	6.8	6.7

In Table X the results of a similar experiment are presented but in this case the substrate used was a 6.0 ppm mixture of glucose and glutamic acid (3.0 ppm each) and the bottle BOD method was used. The data show that the presence of the bicarbonate was responsible for increasing the initial rate of oxygen-uptake between the 24 to 32 hour period compared with the controls using either dilution water. The higher BOD values in the 350 to 480 hour period with standard dilution water represent nitrification.

TABLE X

Effect of 100 ppm Bicarbonate on BOD of 6.0 ppm Glucose-Glutamic Acid

Hours	Standard Dilution Water		Nitrate Dilution Water	
	No bicarbonate ppm	100 ppm bicarbonate ppm	No bicarbonate ppm	100 ppm bicarbonate ppm
4	0	0	0	0
9	0.1	0	0	0
24	0.3	0.3	0	0.3
32	1.0	2.3	0.2	2.4
42	2.5	2.6	2.3	2.5
78	2.6	2.9	2.5	2.7
89	2.9	3.2	2.8	3.3
122	3.3	3.7	2.7	3.6
194	3.9	4.5	3.3	4.1
259	4.3	4.5	3.3	4.3
353	5.4	5.5	4.1	4.5
432	5.8	6.0	4.4	-
456	6.0	-	-	5.4
480	6.5	6.8	4.6	5.3
Initial pH	7.1	7.3	7.2	7.2

C. Warburg BOD Tests

The Warburg procedure, presented as a tentative method in Standard Methods (3), includes the use of a carbon dioxide absorbent (potassium hydroxide) in order to make the direct oxygen-uptake measurement. Since the presence of carbon dioxide has been shown to reduce lag periods with high substrate concentrations such as are used with the Warburg, it was desirable to determine if the use of potassium hydroxide in the center well of the Warburg reaction flask was responsible for early lag in the oxygen-uptake curves. Thus, the validity, or at least the feasibility, of the Warburg Method was questioned.

The immediate problem encountered in this approach was how to set up a range of carbon dioxide concentrations in the Warburg reaction vessels against which the control (with no carbon dioxide) could be compared. The old procedure of eliminating the carbon dioxide absorbent and calculating oxygen-uptake and carbon dioxide concentration was felt to be too inexact and impractical compared with the more recent approach suggested by Pardee (8) and Krebs (9). This involves substituting a so-called "carbon dioxide-buffer" in place of the potassium hydroxide routinely used. The buffer is essentially a solution of diethanolamine which can establish an equilibrium with carbon dioxide so that any new carbon dioxide produced will be absorbed and any carbon dioxide used will be released (through a reversible reaction) back to the flask atmosphere, thus resulting in a constant carbon dioxide tension ambient to the cell-substrate mixture.

Considerable effort was made to apply the procedures recommended by Pardee (8) to the BOD tests on 100 ppm and 1000 ppm glucose using either the common 20 or 150 ml reaction vessels with the small center wells. Various methods of adding carbon dioxide to the system were used, such as flushing the flask with a 5 per cent carbon dioxide-air mixture, partially or completely saturating the diathanolamine just prior to its addition to the reaction flask, and injection of carbon dioxide through rubber tubing connected to the flask. It appeared that injection of a known amount of carbon dioxide gas through a rubber tube after the substrate and diethanolamine were added was the best method of supply. In this way, the changes in gas pressure during the equilibration could be followed more precisely.

The next problem was reduction of the equilibration time in order to set up a constant carbon dioxide-air atmosphere and to begin the oxygen-uptake measurements before oxidation of substrate began. It was found that when the buffer was placed in the center well, the time required for the buffer and the carbon dioxide to reach an equilibrium was 1 to 2 days. This would preclude any oxygen-uptake measurements within the period of major importance. The reason for the long equilibrium time required was the very small surface area of the buffer in contact with the flask atmosphere. The inclusion of strips and cylinders of filter paper in the buffer within the center well increased the surface area and had the effect of reducing the equilibrium time slightly (several hours). When the diethanolamine buffer was placed instead in the outer compartment of the flask (thus with the seed-substrate mixture in the center well) rapid equilibrium was obtained (one-half hour) but oxidation of the substrate was inhibited due to the reduced oxygen diffusion rate to the cells in the center well.

Therefore, it was concluded that the physical design of the standard Warburg reaction vessels as related to surface area and gas diffusion was inimical to the establishment of a system whereby a constant carbon dioxide tension could be obtained. The only solution to this problem would be use of a flask especially designed to furnish sufficient surface area in both reacting compartments. It was felt that the special Dickens-Simer type flask now available would be satisfactory for this purpose. A number of these flasks have been purchased and an investigation of their feasibility begun. However, this being the final phase of the research, results are not available in time for the report. This study will continue and when published, proper credit shall be given to the National Institutes of Health for its support.

III. GENERAL DISCUSSION

It appears that there is a correlation between the observations of Walker regarding carbon dioxide effects on growth of pure cultures and the effects obtained in this work with respect to the effect on substrate catabolism with mixed cultures. The so-called synergistic effect of mixed cultures in attacking even a very simple substrate certainly does not completely preclude a lag period. Since carbon dioxide in these tests reduced (but did not completely eliminate) the lag period with 1000 ppm glucose in a weakly seeded dispersed aeration system, it seems reasonable to conclude that carbon dioxide is at least one of the necessary metabolites required to initiate growth and catabolism. The mechanism of the carbon dioxide effect quite probably resides in its condensation with pyruvate to form citric acid cycle intermediates responsible for the oxidation process.

However, no significant carbon dioxide effect was observed when using the very low substrate concentrations in the standard dilution BOD tests. The cause of the lag periods observed in this system was not definitely determined. It seems conceivable that the necessarily low seed (cell) concentration itself is responsible rather than the amount of metabolite these cells can produce. It would therefore be desirable to extend this work to the use of very large cell inocula (with a low endogenous respiration) in standard BOD tests. Perhaps, with this approach, it would be not only possible to reduce lag periods for a reliable 5-day BOD value, but even to lead to a short-term standard test.

It is felt that the feasibility of Warburg application to sanitary biochemistry is still open to question since carbon dioxide does have a positive effect with high substrate concentrations and since the usual Warburg procedure

eliminates carbon dioxide from the reacting environment. As mentioned previously, investigations of the Warburg direct method will be continued. Meanwhile, the project director has asked for, and received, tentative agreement from the Standard Methods Committee of the Water Pollution Control Association, to hold in abeyance recommendation of the Warburg procedure as a tentative method for BOD determination.

IV. SUMMARY AND CONCLUSIONS

An investigation has been made to determine the effects of carbon dioxide on lag periods observed with mixed microbial cultures in the following systems:

1. Standard BOD tests using low (circa 10 ppm) substrate concentration;
2. Glucose COD catabolism with high (1000 ppm) substrate concentration;
3. Warburg (direct) BOD tests with high substrate concentration.

The following conclusions may be derived from the data obtained in this investigation:

1. The presence of additional carbon dioxide in Standard Dilution BOD tests had no significant effect on early lag periods or on 5-day BOD values. Therefore, it is not recommended that carbon dioxide be added to standard dilution water.
2. With higher substrate concentrations (1000 ppm glucose) absences of carbon dioxide increased the lag period and enrichment with carbon dioxide decreased (but did not completely eliminate) the lag period as determined by the rate of glucose catabolism

represented by COD decrease per unit time. The potential implications of this effect with respect to the Warburg BOD tests have not yet been proven, but the approach to obtain the information has been initiated.

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